

(19) World Intellectual Property Organization  
International Bureau



(43) International Publication Date  
6 February 2003 (06.02.2003)

PCT

(10) International Publication Number  
**WO 03/010204 A1**

(51) International Patent Classification<sup>7</sup>: **C07K 19/00**

(21) International Application Number: PCT/KR02/01416

(22) International Filing Date: 26 July 2002 (26.07.2002)

(25) Filing Language: Korean

(26) Publication Language: English

(30) Priority Data:  
2001-45229 26 July 2001 (26.07.2001) KR  
2002-43968 25 July 2002 (25.07.2002) KR

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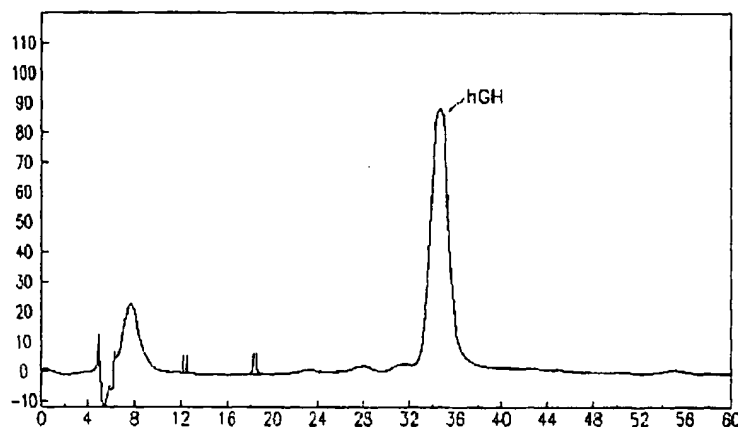
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(81) Designated States (national): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, IL, IN, IS, JP, KE, KG, KP, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZM, ZW.

(84) Designated States (regional): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW),

[Continued on next page]

(54) Title: PROCESS FOR PREPARATION OF POLYPEPTIDES OF INTEREST FROM FUSION PEOLYPEPTIDES



(57) Abstract: The present invention relates to a process of separation a protein of interest comprising expressing the protein of interest fused with a fusion partner, selectively adsorbing on matrix, and performing efficient cleavage reaction on the fusion protein adsorbed on matrix or separated from the matrix, and recovering the protein of interest. Also, the present invention provides a fusion protein comprising a protein of interest and a fusion partner, wherein the fusion partner comprises an amino acid sequence which can be cleaved by ubiquitin cleavage enzyme at its C-terminus, and hwich has a difference in isoelectric point of 1 or more from the protein of interest. According to the present invention, the proteins of interest can be purified in high yield and purity. In addition, complicated separating processes required in the production of recombinant proteins can be eliminated so as to cut down the production cost.

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Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM),  
European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE,  
ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, SK,  
TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ,  
GW, ML, MR, NE, SN, TD, TG).

— *before the expiration of the time limit for amending the  
claims and to be republished in the event of receipt of  
amendments*

**Published:**

— *with international search report*

*For two-letter codes and other abbreviations, refer to the "Guid-  
ance Notes on Codes and Abbreviations" appearing at the begin-  
ning of each regular issue of the PCT Gazette.*

## PROCESS FOR PREPARATION OF POLYPEPTIDES OF INTEREST FROM FUSION POLYPEPTIDES

### BACKGROUND OF THE INVENTION

#### 5 (a) Field of the invention

The present invention relates to a process for separating the protein of interest from fusion protein which comprises a protein of interest and a fusion partner, and a fusion protein useful for the process.

#### (b) Description of the related art

10 As the genetic recombinant technology develops, the proteins which are difficult to obtain from nature can be produced in a large quantity by using fermentation of genetically engineered organism, thereby contributing to the human welfare. Hosts used for the recombinant technologies include *E.coli*, yeast, animal cell, etc. Especially, *E.coli* has the advantages for  
15 protein production in that the gene can be easily manipulated, gives high yield production, and allows use of inexpensive medium.

When a protein of interest is produced by using the recombinant *E.coli*, the protein can be expressed in soluble form or insoluble inclusion body, depending on their properties. The protein expressed in soluble form  
20 has its natural property due to accurate protein folding. The proteins that can coagulate together to be insoluble before forming tertiary structure do not show their natural properties. Thus, the inclusion body of insoluble protein must be solubilized according to various methods and then be refolded. In latter case, the yield and production rate of refolding process  
25 are low. Furthermore, many disulfide bonds in the insoluble protein make accurate refolding difficult. Therefore, it is advantageous to produce the protein of interest in soluble form.

When the protein of interest is expressed in soluble form, it is possible to largely reduce the number of early separation steps such as  
30 centrifugation and filtration by using expanded bed adsorption (EBA) to substantially increase the yield and productivity of the protein. To express

the protein of interest in soluble form, a fusion partner which is capable to be well expressed in soluble form can be fused together with the protein of interest. The examples of fusion partner include GST, maltose binding protein, thioredoxin, ubiquitin, etc. The ubiquitin is a small peptide  
5 consisting of 76 amino acids. When the protein of interest is expressed in a fusion peptide with ubiquitin, the ubiquitin cause the protein to be well expressed in soluble form as well as to increase the expression rate, thereby making the protein in active form.

On the other hand, it is difficult to recover pure protein when the  
10 protein of interest is expressed in soluble form. When the protein of interest is expressed in insoluble inclusion body, it is easy to separate the inclusion body from soluble materials derived from *E.coli* such as the proteins of host cell, DNA, polysaccharides in the early stage of purification. However, it is very difficult to separate soluble protein of interest because it is mixed with  
15 the soluble contaminants such as the proteins of host cell, DNA, polysaccharides. The inclusion body can be solubilized by adding detergents such as urea before disrupting cells in order to purify the inclusion body with EBA, which loses advantage of separating insoluble inclusion body from soluble materials derived from *E.coli* as described above. In conclusion,  
20 when the protein of interest is expressed in a soluble form, or to be applied by EBA process, the protein of interest mixed with DNA, and polysaccharides enters subsequent purification steps. Thus, the efficient purification process is still required.

In typical purification processes, the protein of interest can be  
25 recovered from intracellular proteins by using the difference in charge, solubility, size, hydrophobicity, etc. These purification processes employ inexpensive materials, but have a low selectivity. Thus, to obtain the desired purity, many purification steps comprising various methods are required, and the purification yield decreases largely through the purification  
30 steps. In addition, these methods have a problem wherein the purification steps must be optimized to satisfy the property of the each individual protein of interest. To increase the selectivity of the protein of interest, the affinity

chromatography can be commonly used where an antibody recognizing the unique structure of the protein is immobilized on resin, or a tag with affinity to specific support can be used as a fusion partner. The examples used for increasing selectivity include GST, polyhistidine, etc.(U.S. Patent No. 5,108,919, and Korean Patent No. 177304). In case of affinity chromatography, expensive resin having affinity to the fusion partner or protein of interest restricts industrial application.

In addition, when the protein of interest is expressed in fusion protein, the fusion partner must be removed by cleavage in the subsequent step. In particular, when the protein of interest is intended for medical use, the fusion peptide must be designed to have the suitable cleavage site between the protein of interest and a fusion partner to produce an accurate N-terminus or C-terminus after cleavage, and not to have the cleavage site inside of the protein of interest. The cleavage reaction can be performed with chemical reagents such as acid and CNBr, and proteases such as Factor Xa, and enterokinase. Even though the enzymes have a relatively high selectivity, it is still possible to cleave the other site than intended cleavage site; furthermore, it has a poor efficiency and high cost.

Prior to the present invention, it was developed that a fusion peptide contained a fusion partner which was different in isoelectric point from the protein of interest, and was prepared and separated with ion exchange chromatography. U.S. Patent No. 4,532,207 disclosed that the Arginine tag was fused with anionic EGF, and then purified through cation exchange chromatography to produce EGF. However, the method has a disadvantage in that the charge of a few ionic amino acids directly attached to C-terminus of protein of interest is masked by opposite charge of large protein of interest and so fusion protein cannot adsorb on the matrix efficiently. Thus the method has a low yield of purification.

In U.S. Patent Nos. 5,179,196 and 5,322,930, the fusion peptides consisting of a fusion partner which is different from the protein of interest in the electric property, the protein of interest and CNBR cleavage site and Staph V8 cleavage site, respectively. However, the patents have a problem

in that many proteins having a similar isoelectric point as well as the fusion protein can also adsorb on ion exchange chromatograph, and the cleavage method has a poor selectivity. Thus, the other proteins adsorbed on chromatograph are cleaved and solubilized together with the protein of  
5 interest.

### **SUMMARY OF THE INVENTION**

The present invention provides a DNA construct to separate a protein of interest in high yield conveniently.

The present invention also provides a fusion protein comprising a  
10 protein of interest and a fusion partner to separate a protein of interest in high yield conveniently and efficiently.

The present invention also provides a process for separating a protein of interest in high yield conveniently and efficiently.

The present invention provides a process for separating a protein of  
15 interest by using the change of isoelectric point.

### **BRIEF DESCRIPTION OF THE DRAWINGS**

Fig. 1 shows a schematic tertiary structure of ubiquitin.

Fig. 2 is a photograph of SDS-PAGE analyzing fractions of the fusion  
20 protein eluted on various salt concentration, when the fusion protein is separated by cation exchange chromatography according to Example 3.

Fig. 3 is a photograph of SDS-PAGE analyzing fractions which are obtained by loading on cation exchange chromatograph, and eluting on 400mM of salt concentration after treating with ubiquitin cleavage enzyme  
25 according to Example 5.

Fig. 4 is a photograph of SDS-PAGE analyzing fractions which are obtained by loading on anion exchange chromatograph, and eluting on 400mM of salt concentration after treating with ubiquitin cleavage enzyme according to Example 6.

30 Fig. 5 is a chromatogram analyzing a fraction in lane 3 of Fig.3 with reverse phase HPLC according to Example 7.

Fig. 6 is a photograph of SDS-PAGE showing that the protein of interest can be prepared in high purity by loading cell lysate including a fusion peptide on cation exchange resin, washing by a salt solution with a low salt concentration, and reacting with ubiquitin cleavage enzyme adsorbed on cation exchange resin.

Fig. 7 is a photograph of SDS-PAGE analyzing fractions which are obtained by adsorbing cell extract on cation exchange resin through EBA, precipitating, and reacting with ubiquitin cleavage enzyme in cation exchange resin tower.

Fig. 8 is a photograph of SDS-PAGE analyzing a sample loaded on membrane, a solution eluted by NaCl solution, a UBP cleavage reaction solution after desalting, and fractions obtained by passing the UBP reaction solution through membrane according to Example 11.

Fig. 9 is a photograph of SDS-PAGE analyzing a sample loaded on membrane, a solution eluted by NaCl solution, a UBP cleavage reaction solution after desalting, and fractions obtained by passing the UBP reaction solution through membrane according to Example 13.

#### **DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT**

The present invention relates to a process for separating the protein of interest from fusion protein comprising a protein of interest and a fusion partner, and a fusion protein useful for the process.

More specifically, the present invention provides a fusion protein comprising a protein of interest and a fusion partner, wherein the fusion partner comprises an amino acid sequence to be cleaved with ubiquitin cleavage enzyme on its C-terminus, and wherein the difference of isoelectric point between the protein of interest and the fusion partner is at least 1.

Unless specifically mentioned otherwise, the terms, "protein," "peptide," and "polypeptide" are used interchangeably.

The term, "ubiquitin cleavage enzyme" is intended to mean an enzyme that cleaves a peptide bond next to RGG at C-terminus of protein such as ubiquitin in eukaryotic cell. For example, the enzymes include

UBP1 and UBP2 derived from *Saccharomyces cerevisiae*, UBP41 in muscle cell, etc. As the ubiquitin cleavage enzyme accurately cleaves a peptide bond next to Glycine which is the 76<sup>th</sup> amino acid of ubiquitin, it is possible to generate an accurate N-terminal residue in protein of interest (U.S. Patent  
5 No. 5,847,097).

In the present invention, ubiquitin can be modified to include a tag which comprises 6-10 amino acids selected from a group consisting of His, Lys, and Arg. For examples, the modified ubiquitin can be polyhistidine, polylysine, or polyarginine, etc.

10 The term "protein of interest" is intended to mean a protein to be prepared, and includes physiologically active proteins such as growth hormone, interferon, interleukin, granulocyte colony stimulating factor, erythropoietin, and insulin.

Herein, the term "fusion partner" is intended to mean a peptide which  
15 comprises an amino acid sequence, for example RGG, which can be cleaved by ubiquitin cleavage enzyme in its C-terminus, and which has an isoelectric point difference of 1 or more, preferably 1.5 or more, more preferably 2 or more than that of the protein of interest. The preferred fusion partner is a peptide to make the protein of interest express in soluble form. That is, the  
20 fusion partner may include peptides i) the ubiquitin cleavage site that amino acid sequence can be cleaved by ubiquitin cleavage enzyme at C-terminus such as ubiquitin, a part, and a peptide derived therefrom and, and ii) a peptide or variants thereof to make the fusion protein express in soluble form such as glutathione S transferase (GST), maltose binding protein, or  
25 thioredoxin at N-terminus of the ubiquitin cleavage site.

In an embodiment of the present invention, at least an amino acid in the fusion partner can be substituted, deleted, or inserted to make the difference of isoelectric point at least 1.

The fusion partner can include a peptide where the charge can be  
30 changed by including amino acid sequence which has a different charge from that of the protein of interest at N-terminal or internal site of the fusion partner. The amino acid sequence which has a different charge from that of



the protein of interest can be amino acid sequence consisting of at least a kind of amino acid selected from His, Lys and Arg. Preferably, The amino acid sequence can be a peptide consisting of 2-30 amino acids, for examples polyhistidine, polylysine, and polyarginine.

5           Alternatively, the amino acid sequence which has a different charge from that of the protein of interest can be an amino acid sequence consisting of at least a kind of amino acid selected from glutamic acid and aspartic acid. Preferably, the amino acid sequence can be a peptide consisting of 2-30 amino acids, for examples polyglutamate, polyaspartate, etc. For examples,  
10 the fusion partners can include ubiquitin, parts or variants thereof, and a synthetic peptide comprising ubiquitin or ubiquitin cleavage site, which include amino acid sequence having a different charge from that of the protein of interest at its N-terminus or internal site.

          Herein, the term "amino acid sequence having a different charge from  
15 that of the protein of interest" means an amino acid sequence which has a different charge from that of the protein of interest under the pH condition used in the purification process of the fusion protein or the protein of interest, thereby causing the adsorption difference on matrix between protein of interest and the fusion partner. If pH of the protein of interest is known, it is  
20 possible to design an amino acid sequence to make the charge difference. For examples, when the protein of interest has isoelectric point of 7 or less, amino acid sequence linked continuously at least two positively-charged amino acids such as Lys and Arg can be used. When the protein of interest has isoelectric point of 7 or more, amino acid sequence linked continuously  
25 at least two negatively-charged amino acids such as Glutamic acid and Aspartic acid can be used.

          The amino acid sequence having a different charge from that of the protein of interest can be located at N-terminal or internal site of the fusion partner. It is possible to easily change the adsorption degree of the fusion  
30 partner and the protein of interest on matrix, for examples ion exchange resin or membrane by changing the charge of the fusion partner.

          While most of proteins for medical use have isoelectric point of 7.0 or

less, nucleic acids and endotoxin in cell extract also show a low isoelectric point distribution. Thus, it is possible to separate the fusion protein from contaminants derived from cell by combining the protein of interest with the fusion partner of high pI.

5 For example, because the ubiquitin has pI of 6.56 which is close to a neutral pI value, it is difficult to separate the weak acidic protein of interest from the contaminating materials such as nucleic acids, endotoxin and proteins of host cell by using ion exchange resin or membrane based on the isoelectric point difference. In such case, it is possible to increase pI of the  
10 fusion protein by modifying ubiquitin with cationic peptide tag inserted into the N-terminal or internal site of ubiquitin to produce the fusion protein comprising the modified ubiquitin and the protein of interest. Thus, the fusion protein can be easily separated from the contaminating materials derived from the host cell.

15 In addition, tag including ionic amino acids can be ligated to N-terminus of ubiquitin to induce the isoelectric point difference between the protein of interest and the fusion partner to be cleaved by ubiquitin cleavage enzyme. Furthermore, the ubiquitin can be modified by substituting at least an amino acid of ubiquitin, or by inserting an ionic amino acid therein.

20 The fusion partner of the present invention can be a modified ubiquitin where at least an amino acid located on surface of tertiary structure of ubiquitin can be substituted to have a different charge. For example, at least an amino acid located on surface of tertiary structure of ubiquitin can be substituted with at least an amino acid selected from His, Lys, and Arg, or  
25 with at least an amino acid selected from Glu and Asp.

In reference to the tertiary structure of ubiquitin as shown Fig. 1 (Vijay-Kumar et al., J Mol Biol 194:pp. 531 (1987)), amino acids suitable for being substituted can be selected. The amino acid which can be substituted in the fusion partner satisfies the requirements that the  
30 substitution of the amino acid changes the surface charge of ubiquitin without changing the tertiary structure of ubiquitin in order to make the ubiquitin cleavage enzyme recognize the fusion protein after the substitution.

The amino acid to be substituted can be selected from amino acids which are located on loop but not within secondary structure of alpha-helix and beta-sheet in secondary structure, and which are located on surface of tertiary structure.

- 5           The fusion partner can be selected from modified ubiquitin where the amino acids on at least a site selected from a group of consisting Glu 16, Glu 18, and Glu 64 can be substituted. Preferably, amino acids on Glu 16, Glu 18, and Glu 64 can be substituted with Arg, Lys, and Arg, respectively.

          In the present invention, the term "cell extract" is intended to include  
10 cell lysate, and culture solution which contains the fusion protein comprising the protein of interest.

          The present invention provides a process for separating the protein of interest by using the fusion protein.

          In addition, the present invention provides a process for separating a  
15 protein of interest from a fusion protein comprising:

- a) expressing a fusion protein comprising the protein of interest and the fusion partner in a host cell;
- b) loading the fusion protein on matrix where the fusion partner can adsorb;
- 20 c) treating the adsorbed matrix with ubiquitin cleavage enzyme; and
- d) eluting the cleaved protein of interest from the matrix.

          The present invention also provides a process of separating a protein of interest from a fusion protein comprising:

- a) expressing a fusion protein comprising the protein of interest and  
25 the fusion partner in the host cell;
- b) loading the fusion protein on matrix where the fusion partner can adsorb;
- c) recovering the fusion protein from the matrix;
- d) treating the recovered fusion protein with ubiquitin cleavage  
30 enzyme; and
- e) separating the protein of interest from the fusion partner by using the adsorption difference on matrix between the protein of interest and the

fusion partner.

Among the above steps of separating the protein of interest, the step loading the fusion partner on matrix in step b) can be performed by i) loading the cell extract including the fusion protein on matrix which the fusion partner  
5 can adsorb, or ii) by loading the cell extract including the fusion protein on matrix which the protein of interest can adsorb, recovering the fusion protein from the matrix, and loading the recovered fusion protein on matrix which the fusion partner can adsorb.

The matrix can be an ion exchange resin or a membrane with charge.  
10 The protein of interest and the fusion partner applicable to the present invention can be peptide or protein which has isoelectric point difference of 1 or more, preferably 1.5 or more, more preferably 2 or more. For examples, the protein of interest can be a protein having isoelectric point of 7.0 or more, or 7.0 or less.

15 In one aspect, the present invention provides an efficient process for separating or purifying the protein of interest comprising:

a) expressing a fusion protein comprising a protein of interest, and a fusion partner in host cell, wherein the fusion partner includes an amino acid sequence having a different charge from that of the protein of interest at N-  
20 terminal or internal site, and an amino acid sequence to be cleaved with the ubiquitin cleavage enzyme at C-terminus;

b) loading cell extract containing the fusion protein on matrix where the fusion protein can adsorb;

c) treating the adsorbed matrix with the ubiquitin cleavage enzyme;  
25 and

d) eluting the cleaved protein of interest from the matrix.

In another aspect, the present invention provides an efficient process for separating or purifying the protein of interest comprising:

a) expressing a fusion protein comprising a protein of interest and a  
30 fusion partner in a host cell, wherein the fusion partner includes an amino acid sequence having a different charge from that of the protein of interest at N-terminal or internal site, and an amino acid sequence to be cleaved with

the ubiquitin cleavage enzyme at C-terminus;

b) loading cell extract containing the fusion protein on matrix which the fusion partner can adsorb;

c) recovering the fusion protein from the matrix;

5 d) treating the recovered fusion protein with the ubiquitin cleavage enzyme; and

e) separating the protein of interest from the fusion partner by using the adsorption difference on matrix between the protein of interest and the fusion partner.

10 In an embodiment of the present invention, the present invention provides a process for preparing the protein of interest with pI of 7.0 or less. More specifically, a fusion protein comprising the protein of interest is adsorbed on matrix, and then the fusion protein adsorbed on the ion exchange resin or membrane is directly treated with the ubiquitin cleavage  
15 enzyme without eluting the fusion protein, so as to produce the protein with high purity. This process comprises the steps of expressing the fusion protein comprising the protein of interest with pI of 7.0 or less and the fusion partner where the fusion partner includes an amino acid sequence to be cleaved by ubiquitin cleavage enzyme at C-terminus, and an amino acid  
20 sequence at least two positively-charged amino acids at N-terminal or internal site, loading the cell extract containing the fusion protein on ion exchange resin or membrane which the fusion partner can adsorb, treating the ion exchange resin with the ubiquitin cleavage enzyme, and eluting the protein of interest from the ion exchange resin or membrane.

25 Before treating with the ubiquitin cleavage enzyme, the contaminant proteins, endotoxin and nucleic acid derived from host cell which weakly adsorb the matrix can be dissociated by washing resin tower with salt solution of such a low concentration that the fusion protein cannot dissociate, thereby increasing the purity of the final product. After the enzymatic  
30 cleavage of the fusion protein, the protein of interest can be selectively eluted in high purity by applying a salt solution of such a low concentration that only the protein of interest can be eluted.

In another aspect, the present invention provides a process of preparing the protein of interest with pI of 7.0 or less. More specifically, The process comprises the steps of expressing the fusion protein comprising the protein of interest with pI of 7.0 or less and the fusion partner, where the  
5 fusion partner includes an amino acid sequence to be cleaved by ubiquitin cleavage enzyme at C-terminus and an amino acid sequence at least two positively-charged amino acids at N-terminal or internal site, loading the cell extract containing the fusion protein on cation exchange resin which the fusion partner can adsorb, recovering the fusion protein from the cation  
10 exchange resin or membrane, treating the recovered fusion protein with the ubiquitin cleavage enzyme, and separating the protein of interest by using the adsorption difference on ion exchange resin between the fusion partner and the protein of interest. For example, the step of separating the protein of interest from the fusion partner by using the adsorption difference on  
15 matrix can be performed by loading the fusion protein treated with the ubiquitin cleavage enzyme on anion exchange resin to elute the fusion partner, and then eluting the adsorbed protein of interest after adsorbing the fusion protein, or by loading the fusion protein treated with ubiquitin cleavage enzyme on cation exchange resin to adsorb the fusion partner, and then  
20 eluting the protein of interest.

In the embodiment of the present invention, the present invention provides a process for preparing the protein of interest with pI of 7.0 or more. More specifically, the process comprises the steps of expressing the fusion protein comprising the protein of interest with pI of 7.0 or more and the fusion  
25 partner where the fusion partner includes an amino acid sequence to be cleaved by ubiquitin cleavage enzyme at C-terminus and an amino acid sequence at least two negatively-charged amino acids at N-terminal or internal site, loading the cell extract containing the fusion protein on cation exchange resin which the protein of interest can adsorb, recovering the  
30 fusion protein from the cation exchange resin, loading the fusion protein on anion exchange resin which the fusion partner can adsorb, recovering the fusion protein from the anion exchange resin, treating the recovered fusion

protein with the ubiquitin cleavage enzyme, and separating the protein of interest by using the adsorption difference on anion exchange resin.

In particular, because most of intracellular proteins have isoelectric point close to weak acid pH, the proteins, nucleic acids, and endotoxin in cell  
5 extracts are negatively charged at pH 7.0. According to the present invention, the fusion partner can be modified to include an positively-charged amino acid sequence. Thus, Most of intracellular proteins, nucleic acids, and/or endotoxin can easily be eliminated by passing the cell extract containing the fusion protein through cation exchange resin.

10 The cation exchange resins applicable to the present invention include CM Sepharose, SP Sepharose, etc. The anion exchange resins include Q Sepharose, DEAE Sepharose, etc.

In the present invention, cell extract of host obtained after the production of the fusion protein can be made to directly enter the expanded  
15 bed absorption step, so as to reduce the number of early separation steps such as centrifugation and filtration, and to perform the adsorption and cleavage in a step. The expanded bed adsorption chromatography can remove solid contaminants from cell extract, and provide the desired protein in a step, so that the chromatography can replace initial separation process  
20 such as centrifugation and filtration of existing production method.

The purity of the protein of interest can be measured with reverse-phase HPLC. According to the process of the present invention, the amounts of DNA and endotoxin mixed in the protein of interest can decrease.

In the present invention, various proteins can be fused to the fusion  
25 partner and expressed in the suitable prokaryotic and eukaryotic cell by using a suitable expression system.

The invention is further illustrated by the following Examples. The following examples are intended only to illustrate the invention and are not intended to limit the scope of the invention as recited by the claims.

### Example 1: Producing expression vector for K6Ub-hGH

A gene encoding human growth hormone(Genbank Accession No. K02382, Human growth hormone synthetic gene, complete CDS) was cloned into pGNX2 vector (Korean Patent Publication No. 0319520) to produce  
5 pGNX2hGH. 400-bp of product was amplified by using the pGNX2hGH as a template and hGHN and hGHCB (Table 1) as primers, and then cleaved by BamH1.

A gene encoding ubiquitin was amplified by using a gene of Genbank Accession No. M17524(Synthetic human ubiquitin gene, complete cds) as a  
10 template and a forward primer (5'-GCAGCATATGCAGATTTTCGTC-3'(UBF) and a backward primer (5'-CGACGGCGCCACCTCTTAGCC-3'(UBR)) through PCR, and then 230-bp of amplification product was cleaved by NdeI. The resultant product was ligated with pUC18 which was cleaved with HindIII to produce pUC18Ubq. To fuse hGH gene, the pUC18Ubq was cleaved  
15 with sfo1 and BamH1, and then was fused with 400-bp of PCR product to produce pUC18ubhGH. To transfer pUC18ubhGH to the expression vector, pGNX4(Korean Patent Publication No. 0319520) and pUC18ubhGH were cleaved by Nde1 and BamH1, and then ligated to produce pGNX4ubhGH. *E.coli* XL1-Blue MR was transformed by pGNX4ubhGH, screened in the  
20 medium containing Kanamycin, and then cultured in LB liquid medium. At cell density of  $OD_{600}=0.6$ , the expression of human growth hormone in the cell was induced by addition of 0.5 mM IPTG, and the expression was confirmed by SDS-PAGE analysis.

To fuse 6 lysine tag to the N-terminus of fusion protein, a strand of  
25 lysine tag, KTT (Table 1) was synthesized and amplified by using 5'-primer(KTN) and 3'-primer(KTC) through PCR to obtain 45-bp of PCR product. The PCR product was cleaved by Nde1(NEB, England) and Ase1(NEB, England), and fused with pGNX4UbhGH cleaved with NdeI to produce pGNX4K6UbhGH. *E.coli* XL1-Blue MR was transformed by  
30 pGNX4K6UbhGH, screened in the medium containing Kanamycin, and then cultured in LB liquid medium with shaking. The expression of human



growth hormone in the cell was induced by addition of 1 mM IPTG, and the expression was confirmed by SDS-PAGE analysis. The sequence analysis of the expressed plasmid DNA confirmed that 6 lysine tag at N-terminus was encoded by the codons of AAG AAA AAA AAG AAA AAG.

5

### **Example 2: Producing expression vector for K10-UBP**

To clone the UB1 gene, the genome of *S.cerevisiae* ATCC 208275 was used as a template and amplified by using the primers of UBPN and UBPC(Table 1) through PCR to produce 2400-bp of UB1 gene. The UB1  
10 1 was cloned between NdeI and BamHI site of pRSETc vector. The cloned vector and pGNX4 vector were cleaved with NdeI and BamHI and fused to obtain pGNX4UB1. *E.coli* XL1-Blue MR was transformed by pGNX4UB1, screened in the medium containing Kanamycin, and then cultured in LB liquid medium containing Kanamycin. At the cell density of  $OD_{600}=0.6$ , the  
15 expression of UB1 in the cell was induced by addition of 0.5 mM IPTG. The activity of UB1 was determined by cleaving the fusion protein of Example 1 with the UB1.

To easily purify with maintaining the UB1 activity, the 10 lysine tag was ligated to the N-terminus of the fusion peptide. 10K(Table 1) of the  
20 oligonucleotide having 10 AAG codons was synthesized, and the NdeI and AseI sites in the 10K were cleaved by the restriction enzymes, and then fused with pGNX4UB1 cleaved with NdeI and CIP to produce pGNX410KUB1. The activity of UB1 was determined with the same method as pGNX4UB1.

25 The primer sequences used in Examples 1 and 2 are shown in the following Table 1.

[Table 1]

Primer	Nucleic acid sequence(5' ( 3')	SEQ ID NO.
UBF	5'-GCAGCATATGCAGATTTTCGTC-3'	1
UBR	5'-CGACGGCGCCACCTCTTAGCC-3'	2
hGHN	5'-TTTCCAACCATTCCACTG-3'	3
hGHCB	5'-GACTGGATCCTTAAAAACCACAAGAACC-3'	4
KTN	5'-GGGGAATTCCATATG-3'	5
KTT	5'- GGGGAATTCCATATGAARAARAARAARAATAAT GGATCCCCC-3'	6
KTC	5'-GGGGGATCCATTAAT-3'	7
UBPN	5'- CTACCGCGGTTCATATGGCTTTTGTTTATTGAAAGA AG-3'	8
UBPC	5'-ATTGGATCCTTAGTTTACATCTTTACC-3'	9
10K	5'-GGCCATATG(AAG)10ATTAATGGCC-3'	10

Note: R is Adenine or Guanine

### 5 Example 3: Producing and Separating the fusion protein

E. coli TG1 was transformed with the expression vector pGNX4K6UbhGH obtained in Example 1 to produce transformant colony. The transformant colony was inoculated in 5 mL of LB medium containing 50 mg/L of Kanamycin, and cultured at 37 °C. 1 mL of cultured transformant  
10 was inoculated in 100 mL of R media containing 5 g/L glucose (the composition of R media is shown at Table 1) and cultured at 30°C. The resultant culture was inoculated in 1.4L of R media containing 15g/L glucose, and cultured according to fed-batch culture method where the 500g/L of glucose solution was added at the time of glucose amount of 0 g/L. When  
15 the Absorbance of culture solution reached about 70, the expression of

protein was induced by addition of 1 mM IPTG. The cell was harvested with centrifugation, suspended in 20 mM of phosphate buffer solution(pH 7.5), and then disrupted with microfluidizer. The cell extract was centrifuged at 15,000 rpm for 60 minutes to produce supernatant of soluble cell extract, and  
 5 the soluble cell extract was separated with ion exchange resin.

[Table 2]

ingredient	Added amount
$\text{KH}_2\text{PO}_4$	3 g/L
$\text{K}_2\text{HPO}_4$	3 g/L
$(\text{NH}_4)_2\text{SO}_4$	4 g/L
Trisodium citrate	2.3 g/L
Casamino acid	2 g/L
Glucose	5 g/L
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.22 ml/L
Trace metal solution	1 ml/L
Kanamycin	100 mg/L
Thiamine	5 mg/L

Econo-Pack column (Bio-Rad, USA) was filed with 2mL of SP-Sepharose Fast Flow cation exchange resin (Pharmacia, Sweden), and  
 10 operated at 1 mL/min. Phosphate buffer solution(pH 7.5) was used as a mobile phase, and column was equilibrated with 15 bed volumes of phosphate butter before loading sample. The column was loaded with the supernatant solution, washed with 3 bed volumes of buffer solution, and eluted by various solutions containing the same buffer substance of mobile  
 15 phase and NaCl in various concentration. Namely, 6 ml of buffer solutions with NaCl concentration of 200, 400, 600, 800, and 1000mM were used respectively, and the column was washed with 6 bed volumes of 1 M NaCl buffer solution after elution. The fractions obtained by using the elution solution with the various salt concentration were analyzed by SDS-PAGE,  
 20 and the result was shown in Fig.2. (SM: Molecular weight marker, 1: a flow through fraction, 2: a solution eluted by buffer solution, 3: a solution eluted by a buffer solution with 200 mM NaCl, 4: a solution eluted by a buffer solution with 400 mM NaCl, 5: a solution eluted by a buffer solution with 600 mM

NaCl, 6: a solution eluted by a buffer solution with 800 mM NaCl, 7: a solution eluted by a buffer solution with 1M NaCl ).

As shown in Fig.2, the fusion protein, K6Ub-hGH was eluted on 400mM of salt concentration, so as to remove most of contaminant proteins  
5 derived from *E.coli*. However, the fusion protein was adsorbed with various cationic proteins.

#### **Example 4: Producing ubiquitin cleavage enzyme**

*E. coli* TG1 was transformed with the expression vector,  
10 pGNX4K10UBP1 produced in Example 2, and recombinant *E.coli* was cultured according to the method of Example 3 to produce ubiquitin cleavage enzyme fused with 10 lysine tag (K10-UBP). According to substantially the same method of Example 3, the cell extract of recombinant *E.coli* was obtained, and the enzyme was separated by using a cation exchange  
15 chromatography. In Example 1, the K6Ub-hGH was eluted by a buffer solution with 400mM of salt concentration (K6Ub-hGH 400mM fraction), and in this Example, K10-UBP was also present in a fraction obtained on 400mM of salt concentration (K10-UBP 400mM fraction). Accordingly, the solutions containing the two fusion proteins could be adjusted to have the same ionic  
20 strength.

#### **Example 5: Separating process by using ubiquitin cleavage reaction and cation exchange resin**

The K6Ub-hGH 400mM fraction partially separated in Example 3, and  
25 K10-UBP 400mM fraction partially separated in Example 4 were mixed in the ratio of 50 : 1, cleaved at 30 °C for 1 hour, and analyzed by SDS-PAGE(Fig. 3, lane 2).

The reaction solution was diluted with 20 mM phosphate buffer solution(pH 7.5) in the ratio of 1:1 to lower the ionic strength, and loaded on  
30 SP-Sepharose Fast Flow cation exchange resin column. The human growth hormone was directly eluted without being adsorbed on the resin. Other proteins were adsorbed on the cation exchange chromatograph, so as

to separate the human growth hormone in high yield and purity(Fig 3, lane 1: K6Ub-hGH 400mM elution fraction, 2: after treating K6Ub-hGH 400mM elution fraction with UBP, 3: a flow through solution obtained by eluting the K6Ub-hGH 400mM solution after treating with UBP, 4: a solution washed by  
5 buffer solution, 5: a solution washed by a buffer solution with 200mM NaCl, 6: a solution washed by a buffer solution with 1M NaCl).

**Example 6: Separating process by using the ubiquitin cleavage reaction and anion exchange resin**

10 The K6Ub-hGH 400mM fraction partially separated in Example 3, and The K10-UBP 400mM fraction partially separated in Example 4 were mixed in the ratio of 50:1, cleaved at 30°C for 1 hour, and desalted by PD-10 column(Pharmacia) to well adsorb the human growth hormone on anion exchange chromatograph. The desalted reaction solution was loaded on Q-  
15 Sepharose Fast Flow anion exchange resin column, and washed with 20mM phosphate buffer solution to remove unadsorbed proteins. The human growth hormone was eluted by 200mM salt solution (Fig. 4, lane 1: a solution obtained by treating K6Ub-hGH 400mM fraction with UBP, 2: a eluted solution obtained by loading the K6Ub-hGH 400mM after treating with  
20 UBP, 4: a solution washed by a buffer solution with 50mM NaCl, 5: a solution washed by a buffer solution with 200mM NaCl, 6: a solution washed by a buffer solution with 1M NaCl).

**Example 7: Analysis with reverse-phase HPLC**

25 To determine the purity of human growth hormone obtained by Example 5, the reverse-phase HPLC was performed. The fraction obtained from the fraction on lane 3 of Fig.3 was used as a sample. The column was Vydac®C4 (300A, 5um, 4.6mm id x 250mm, USA). The analyzing condition was decided according to the analyzing condition of human growth hormone  
30 as described on European Pharmacopeia (European Pharmacopeia 1999: 0951). The temperature of column was maintained at 45°C, and 29% isopropanol and 71% 50mM Tris buffer solution(pH 7.5) was used as a

mobile phase. The flow rate was 250  $\mu\text{l}/\text{min}$ . As shown in Fig. 5, the human growth hormone with purity of 99% or more was obtained by using two purification steps of ion exchange resin.

#### 5           **Example 8: cleavage reaction in resin column**

SP-Sepharose Fast Flow cation exchange column was set according to Example 3, equalized with a phosphate buffer solution (pH 7.5), and then 3mL of K6UbhGH 400mM fraction was diluted with the same volume of buffer solution to lower the ionic strength, and injected into the column. The  
10 unadsorbed proteins were removed by washing with 6 mL of the buffer solution. 1 mL of a fraction containing K10-UBP 400mM fraction was diluted with the same volume of buffer solution, injected into the resin tower, and reacted for 1 hour at room temperature. After the reaction, the cleaved human growth hormone was eluted by pouring 6mL of buffer solution with  
15 200mM NaCl, and then the remaining adsorbed proteins was eluted by pouring 6 mL of buffer solution with 1M NaCl. The fractions obtained from each step were analyzed by SDS-PAGE to show the result in Fig.6(SM: molecular weight marker 1: K6UbhGH 400mM fraction, 2: a solution washed by buffer solution, 3: a solution eluted after loading K10-UBP 400mM fraction,  
20 4: a solution washed by a buffer solution with 200mM NaCl, 5: a solution washed by a buffer solution with 1M NaCl).

The proteins contained in the K6Ub-hGH 400mM fraction adsorbed well on the cation exchange resin, and thus did not elute during pre-washing step by using the buffer solution (lane 2). Like this, the proteins contained  
25 in the K10-UBP 400mM fraction adsorbed well (lane 3). After the cleavage reaction with ubiquitin cleavage enzyme, the human growth hormone was released from the fusion protein. After cleavage reaction, the tower was poured with a buffer solution with 200mM NaCl, and then the cleaved human growth hormone was present in the eluted fraction in high purity (lane 4).

30

#### **Example 9: Applying EBA to separating process**

STREAMLINE® 25 filled with 100ml of STREAMLINE® SP resin was

equalized with the phosphate buffer solution (pH 7.5), and then injected from down to upward by cell extract(400ml) containing K6Ub-hGH fusion protein obtained according to Example 3, so that expansion ratio of column bed is 2. After injection of the sample, the column was washed with phosphate buffer  
5 solution at 2 times by volume of resin to remove the solid materials such as cell debris, DNA, and cell proteins. To cleave the human growth hormone from fusion protein adsorbed on the resin, the resin was precipitated, and 50ml of K10-UBP solution obtained in Example 4 was diluted with buffer solution and then was injected into the column. The reaction was  
10 performed by circulating the solution in column with peristaltic pump, and the processing degree of cleavage reaction was determined by measuring an absorbance with spectrophotometer. As the reaction processed, the human growth hormone cleaved from the absorbed fusion protein was released increasingly, and after 3 hours, no increase of absorbance  
15 confirmed the termination of the reaction. After the reaction, the released human growth hormone was eluted by pouring a buffer solution with 100mM NaCl, and was washed with solutions containing 600mM, and 1M NaCl, respectively (Fig. 7, lane 1: molecular weight marker, 2: cell lysate, 3: a solution washed after injecting the cell lysate, 4: a solution eluted after  
20 treatment of UBP, 5: a solution washed by a buffer solution with 100mM NaCl, 6: a solution washed by a buffer solution with 600mM NaCl, 7: a solution washed by a buffer solution with 1M NaCl).

**Example 10: Producing expression vector of**  
25 **pGNX4K6UbIFNalpha-2b**

To fuse the ubiquitin protein and the interferon protein, two step PCR was performed.

In the first step, the ubiquitin gene was amplified by using the vector including ubiquitin coding gene(Genbank Accession No. M17524, Synthetic  
30 human ubiquitin gene, complete cds) as a template and F6KUb (containing Nde I site, 6 lysine, 5'-terminus of ubiquitin as shown in Table 3) and ORIFN (containing 3'-terminus of ubiquitin and 5'-terminus of human interferon) as

primers to produce 250-bp and 500-bp of PCR products, respectively.

In the second step, to fuse the two PCR products, the PCR products as templates were amplified with primer F6KUb (containing *Nde* I site, 6 Lysine, and 5'-terminus of ubiquitin) and primer RIFN(containing 3'-terminus of human interferon, and *Bam* HI site) to produce 750-bp of PCR product. The resultant product and pGNX4 were cut by *Nde*I and *Bam* HI, and ligated to produce pGNX4K6UbIFNalpha-2. The nucleic acid sequence analysis of the vector confirmed that 6 lysine tag, ubiquitin, and human interferon were expressed in a protein.

*E.coli* TG1 was transformed by pGNX4K6UbIFNalpha-2b, and the expression of the fusion peptide was induced by addition of 0.5mM IPTG, and analyzed with SDS-PAGE. The primer sequences used in the Example were shown in Table 3.

[Table 3]

Primer	Nucleic acid(5'→ 3')	Note	SEQ ID NO.
F6Kub	5'-GGGTAAACATATGGAGGATGA GGATGAAGACAAGATTTTCGT CAAGAC-3'	<i>Nde</i> I site, ATG, and 6 lysine tag	11
ORIFN	5'-AGGGAGATCACAGCCACCTCT TAGCCTTAGCACA-3'	-	12
OFIFN	5'-TAAGAGGTGGCTGTGATCTCC CTGAGACCCACA-3'	-	13
RIFN	5'-GCGCGGATCCTTATTCCTTCC TCCTTAATCTT-3'	TAG and <i>Bam</i> HI site	14

#### Example 11: Separating interferon with cationic membrane

The *E.coli* TG1 was transformed with pGNX4K6UbIFNalpha-2b, and the clone expressing the fusion protein was selected. The clone was cultured in 5ml of LB medium and inoculated in 100ml of LB medium. At the absorbance of 1.5-2, the expression of interferon was induced by addition of IPTG, and the cell was harvested at 4 hours after adding IPTG. The cell was recovered by centrifuging at 1300 rpm for 3 minutes,



suspended in 4ml of 20mM phosphate buffer solution, and then disrupted by ultrasonicator. The supernatant was obtained by centrifuging cell lysate at 1300rpm for 30 minutes for use in following separation process.

Vivapure S mini H (Vivascience, Germany) cation exchange  
5 membrane was used after being equalized with 20mM phosphate buffer solution for purifying the interferon. The supernatant solution containing interferon was loaded on the membrane, and adsorbed by centrifuging at 1,200g. The membrane was washed twice with 0.4ml of phosphate buffer solution, and eluted by phosphate buffer solution with 1M NaCl. 0.1ml of  
10 the eluted fractions was desalted with Dialysis apparatus (Slide-A-Lyzer mini, Pierce, USA) having cut-off of 3500 for 1 hour, and treated with UBP1. After cleavage reaction for 2 hours, the reaction solution was loaded on the membrane equalized with 20mM phosphate buffer solution, and centrifuged at 1200g for 5 minutes. The interferon was present in the fraction passing  
15 the membrane without adsorbing in high purity (Fig. 8, lane 1: Loading sample diluted in the ration of 1/10, 2: a solution eluted by a buffer solution with 1M NaCl, 3: a UBP cleavage reaction solution after desalting, 4: a fraction passing the membrane after the cleavage reaction).

#### 20       **Example 12: modifying Ubiquitin**

To change the surface charge of the ubiquitin with maintaining the tertiary structure to enable ubiquitin cleavage enzyme to recognize ubiquitin-fusion protein, anionic glutamates on 16 position, 18 position, and 64 position were substituted with arginine or lysine to change the isoelectric  
25 point, in consideration of the tertiary structure of ubiquitin. To modify the ubiquitin, two step-PCR were performed.

In the first step of PCR, the primers having different DNA sequence at the target site were used. Namely, primer FMUB1 was used for modifying the glutamate on 16 position, and 18 position of ubiquitin, primer  
30 RMUB1 was used for modifying glutamate on 64 position of ubiquitin. The PCR product was amplified by using forward primer of FMUB2(containing ATG and Nde I site) and backward primer of RMUB2(containing Sfo I site) to

produce the modified ubiquitin gene. The modified ubiquitin gene was cloned into pGEM-T easy vector to produce pGEM-Mub vector. The sequence analysis of the vector confirmed that the desired modified ubiquitin was obtained.

5           The second step of PCR was performed to fuse the modified ubiquitin gene and human growth hormone gene. The modified ubiquitin was amplified by using the pGEM-Mub as a template, and primer FMUB2 (containing *Nde* I site, ATG, and 5'-terminus of the modified ubiquitin) and primer ORhGH (containing 3'-terminus of the modified ubiquitin and 5'-  
10 terminus of human growth hormone) to produce 250-bp of PCR product.

          The human growth hormone gene was amplified by using a vector including a gene encoding human growth hormone (Genbank Accession no. K02382, Human growth hormone synthetic gene, complete cds) as a template, and primer OFhGH (containing 3'-terminus of the modified ubiquitin,  
15 and 5'-terminus of human growth hormone) and primer RhGH (containing 3'-terminus of human growth hormone and *Bam* HI site) to produce 600-bp of PCR product. To fuse the PCR products, the PCR products as a template was amplified with primers of FMUB2 (containing *Nde* I site and 5'-terminus of modified ubiquitin) and RhGH (containing 3'-terminus of human growth  
20 hormone, and *Bam* HI site) to produce 900-bp of PCR product. The fused PCR product and the expression vector of pGNX4 were cleaved by *Nde* I and *Bam* HI, and ligated to produce pGNX4MUbhGH vector. The nucleic acid sequence analysis of the vector confirmed that the modified ubiquitin was fused with the human growth hormone.

25

### **Example 13: Separating human growth hormone by using the modified ubiquitin**

          E.coli was transformed with pGNX4MUbhGH vector, and the clone expressing the fusion protein was selected. The clone was pre-cultured in  
30 5ml of LB medium and 3% of culture solution was inoculated in 100ml of LB medium. When the absorbance was 1.5-2, the expression of the fusion protein was induced by adding IPTG, and the cells were harvested at 4

hours after addition of IPTG. The cell was recovered by centrifuging at 13,000 rpm for 3 minutes, suspended in 20mM MES buffer to be 50 of absorbance, and disrupted with ultrasonicator. The supernatant solution was obtained by centrifuging cell lysate at 13,000 rpm for 30 minutes, and  
 5 then used for following separating steps.

Vivapure S mini H (Vivascience, Germany) cation exchange membrane was used after being equalized with 20mM MES buffer solution for purifying the fusion protein. The supernatant solution containing the fusion protein was loaded on the membrane, and adsorbed by centrifuging at  
 10 900g. The membrane was washed twice with 0.4ml of MES buffer solution, and eluted by MES buffer solution with 1M NaCl. 0.1ml of the eluted fractions was desalted with Dialysis apparatus (Slide-A-Lyzer mini, Pierce, USA) having molecular cut-off of 3500 for 1 hour, and treated with UBP1. After cleavage reaction for 2 hours, the reaction solution was loaded on the  
 15 membrane equalized with 20mM MES buffer solution, and centrifuged at 900g for 5 minutes to produce the purified human growth hormone.

Like the ionic tag fused to N-terminus of ubiquitin, the isoelectric point increased by replacing amino acids in the internal site of ubiquitin, thereby making the modified ubiquitin adsorb on cationic exchange resin and be  
 20 recognized by ubiquitin cleavage enzyme(Fig. 9, lane 1: Loading sample diluted in the ratio of 1/10, 2: a solution eluted by a buffer solution with 1M NaCl, 3: a fraction obtained by eluting UBP cleavage reaction after desalting, 4: a fraction obtained by passing membrane after the UBP cleavage reaction). The primers were shown in Table 4, where the underlined parts  
 25 of nucleic acid sequence represented the modified parts.

[Table 4]

Primer	Nucleic acid sequence	Note	SEQ ID NO
FMUB1	5'-CTT TGA CCG GTA AAA CCA TAA CAT TGC <u>GCG</u> TTA AAT CTT CCC ATA CC-3'	Amino acids modified at Glu16, Arg16, Glu18, and Lys18	15

FMUB2	5'-GGC CGC ATA <u>TGC</u> AGA TTT TCG TCA AGA CTT TGA CCG GTA AAA CC- 3'	<i>Nde</i> I site, and ATG	16
RMUB1	5'-CTC TTA GCC TTA GCA CAA GAT GTA AGG TGG <u>AGC</u> <u>GCT</u> TCT GAA TGT TG-3'	Amino acid modified at Glu64, and Arg64	17
RMUB2	5'-CGC GGA TCC AGT GGA ATG GTT GGG <u>GCG</u> <u>CCA</u> CCT CTT AGC CTT AGC AC-3'	<i>Sfo</i> I site	18
OrhGH	5'-CAG TGG AAT GGT TGG AAA GCC ACC TCT TAG CCT TAG-3'		19
OfhGH	5'-CTA AGG CTA AGA GGT GGC TTT CCA ACC ATT CCA CTG-3'		20
RhGH	5'-GCC GGA TCC TTA AAA ACC ACA AGA ACC-3'	<i>Bam</i> HI site	21

The protein of interest could be separated easily and economically according to the present invention.

**WHAT IS CALIMED IS:**

1. A fusion protein comprising a protein of interest and a fusion partner, wherein the fusion partner comprises an amino acid sequence at its C-terminus which can be cleaved by ubiquitin cleavage enzyme, and has a difference in isoelectric point of 1 or more from the protein of interest.
2. The fusion protein according to Claim 1, wherein the fusion partner is ubiquitin or a peptide derived from ubiquitin.
3. The fusion protein according to Claim 1, wherein the fusion partner comprises ubiquitin cleavage site at its C-terminus which can be cleaved by ubiquitin cleavage enzyme, and a peptide or its variants selected from a group consisting of Glutathione S transferase(GST), maltose binding protein, and thioredoxin at N-terminus of the ubiquitin cleavage site.
4. The fusion protein according to Claim 3, wherein the ubiquitin cleavage site is ubiquitin or a peptide derived from ubiquitin.
5. The fusion protein according to Claim 1, wherein the fusion partner is a peptide having a charge changed by replacing, inserting, or deleting at least an amino acid in the fusion partner.
6. The fusion protein according to Claim 5, wherein the fusion partner is a peptide having a charge changed by containing an amino acid sequence having a different charge from that of the protein of interest at N-terminal or internal site of the fusion partner.
7. The fusion protein according to Claim 6, wherein the amino acid sequence having a different charge from that of the protein of interest is an amino acid sequence of 2-30 amino acids which include at least a kind of amino acid selected from the group consisting of histidine, lysine, and

arginine.

8. The fusion protein according to Claim 6, wherein the amino acid sequence having a different charge from that of the protein of interest is  
5 an amino acid sequence of 2-30 amino acids which include at least a kind of amino acid selected from the group consisting of glutamic acid and aspartic acid.

9. The fusion protein according to Claim 5, wherein the fusion  
10 partner is a peptide having a charge changed by replacing at least an amino acid on a surface of tertiary structure of ubiquitin.

10. The fusion protein according to Claim 9, wherein at least an amino acid on the surface of tertiary structure of ubiquitin is replaced by at  
15 least an amino acid selected from the group consisting of histidine, lysine, and arginine.

11. The fusion protein according to Claim 9, wherein the fusion partner comprises at least an amino acid replaced at one or more amino acid  
20 position selected from a group consisting of Glu16, Glu18, and Glu64 of ubiquitin.

12. The fusion protein according to Claim 11, wherein the fusion partner is a peptide where Glu16 is replaced by Arg, Glu18 is replaced by  
25 Lys, and Glu64 is replaced by Arg of the ubiquitin.

13. The fusion protein according to Claim 9, wherein at least an amino acid on surface of tertiary structure of ubiquitin is replaced by at least an amino acid selected from the group consisting of glutamic acid and  
30 aspartic acid.

14. The fusion protein according to Claim 1, wherein the protein

of interest is selected from the group consisting of human growth hormone, interferon, interleukin, granulocyte colony stimulating factor, erythropoietin, and insulin.

5           15.     A process of separating a protein of interest from a fusion protein comprising:

          a) expressing the fusion protein comprising the protein of interest and the fusion partner according to claim 1 in a host cell;

          b) loading the fusion protein on matrix which the fusion partner can  
10    adsorb;

          c) treating the adsorbed matrix with ubiquitin cleavage enzyme; and

          d) eluting the cleaved protein of interest from the matrix.

          16.     The process according to Claim 15, wherein the fusion  
15    protein is a fusion protein defined in any one claim of claims 2-14.

          17.     A process of separating a protein of interest from a fusion protein comprising:

          a) expressing the fusion protein comprising the protein of interest and  
20    the fusion partner according to claim 1 in a host cell;

          b) loading the fusion protein on matrix which the fusion partner can adsorb;

          c) recovering the fusion protein from the matrix;

          d) treating the recovered fusion protein with ubiquitin cleavage  
25    enzyme; and

          d) separating the protein of interest from the fusion partner by using the adsorption difference on the matrix.

          18.     The process according to Claim 17, wherein the fusion  
30    protein is a fusion protein defined in any one claim of claims 2-14.

          19.     The process according to Claim 15 or 17, the step loading

the fusion protein on matrix in step b) is performed i) by directly loading cell extract containing the fusion protein on matrix which the fusion partner can adsorb, or ii) by loading cell extract containing the fusion protein on matrix which the protein of interest can adsorb, recovering the fusion protein from  
5 the matrix, and loading the recovered fusion protein on matrix which the fusion partner can adsorb.

20. The process according to Claim 15 or 17, wherein the matrix is an ion exchange resin or a membrane with charge.

10

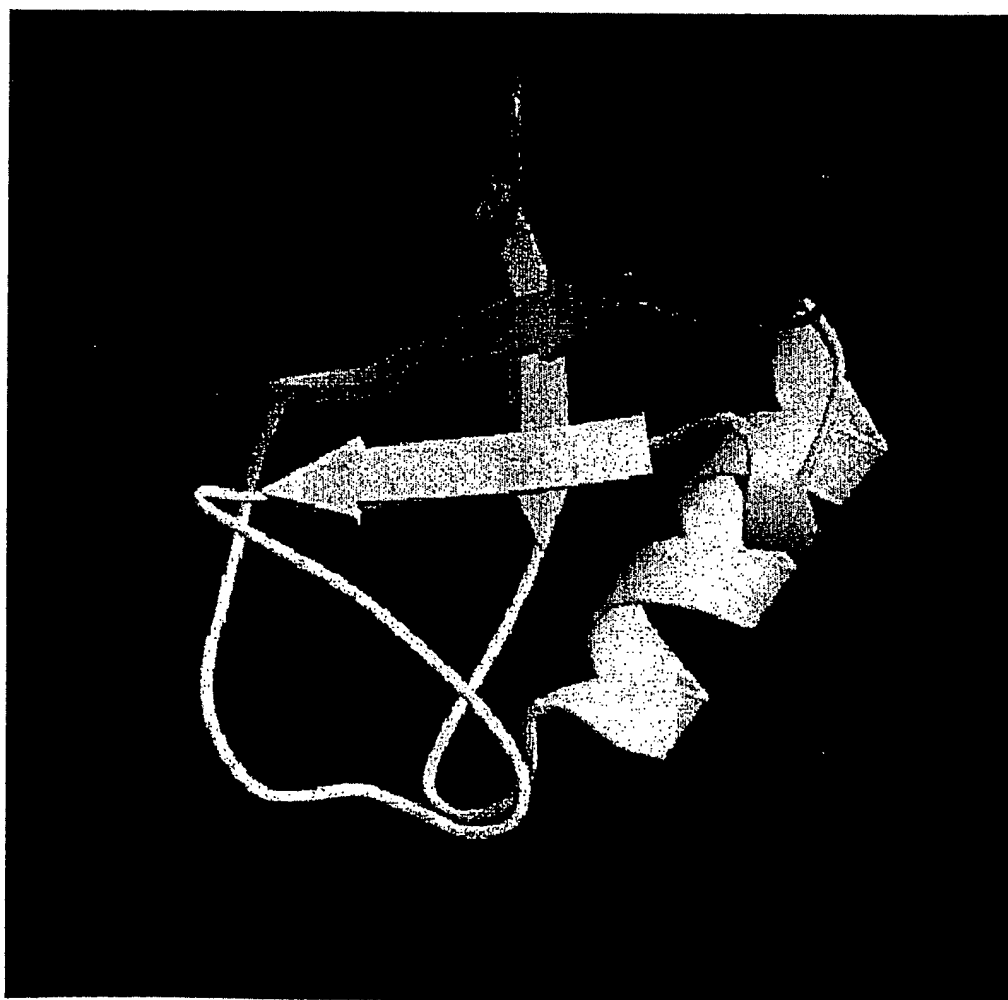
21. The process according to Claim 15 or 17, wherein the protein of interest has pI of 7.0 or less.

22. The process according to Claim 15 or 17, wherein the  
15 protein of interest has pI of 7.0 or more.

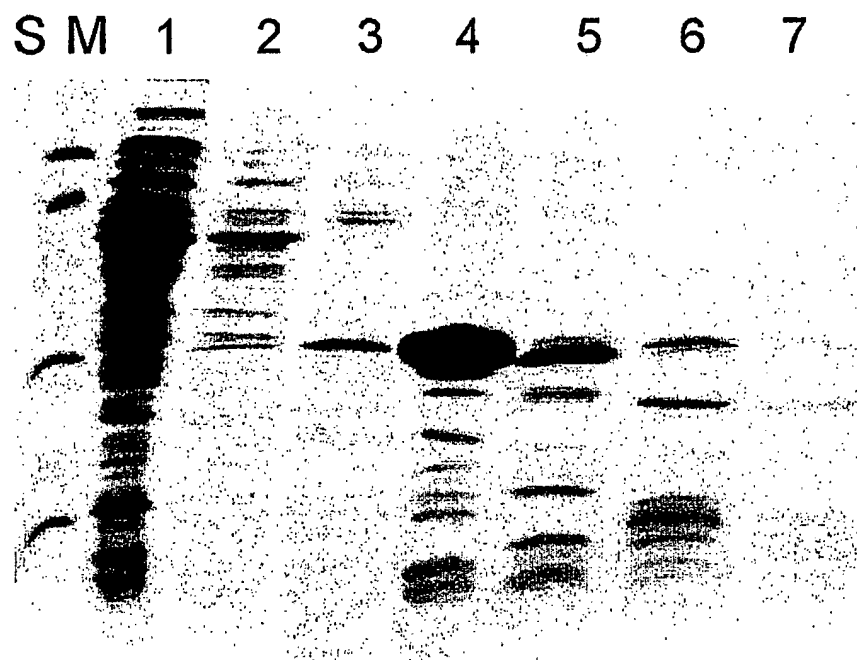


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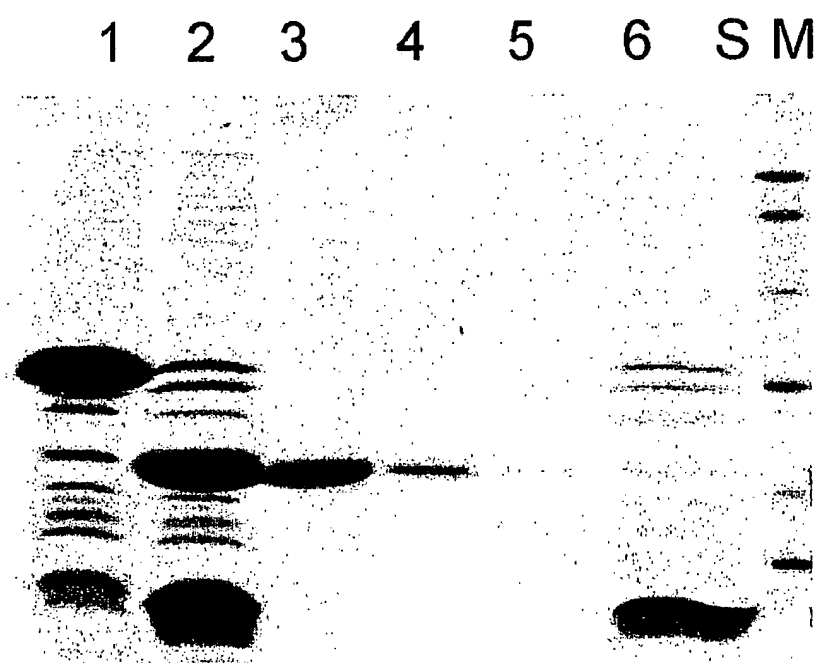
**FIG.1**



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**FIG.2**

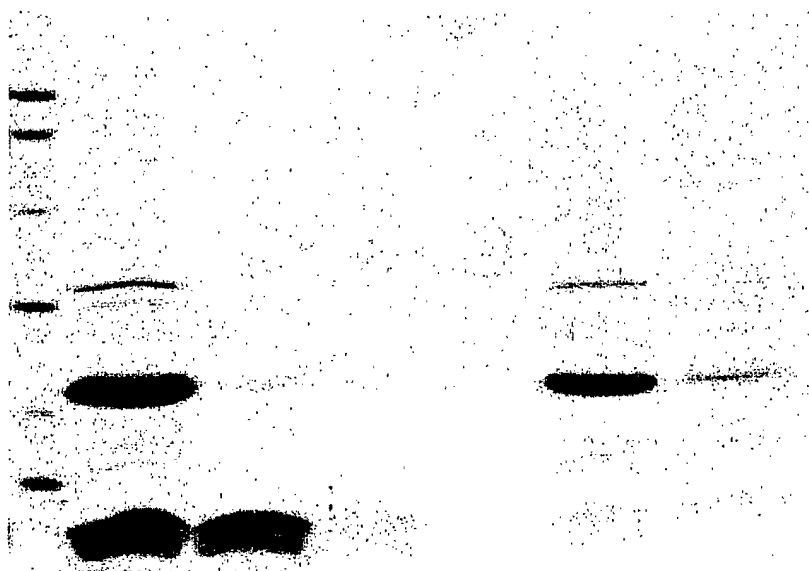
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**FIG.3**

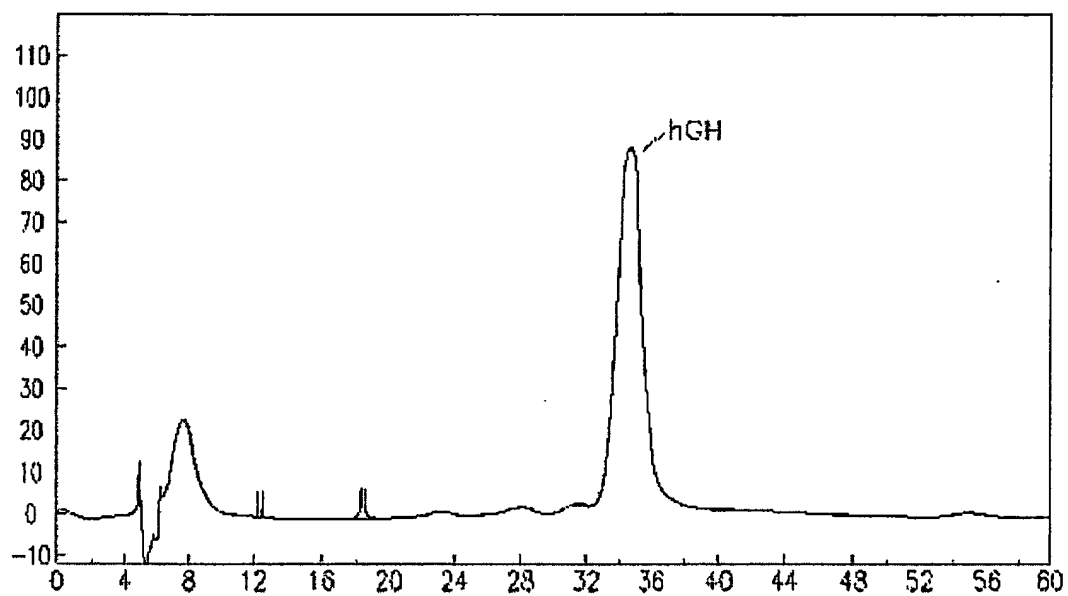
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**FIG.4**

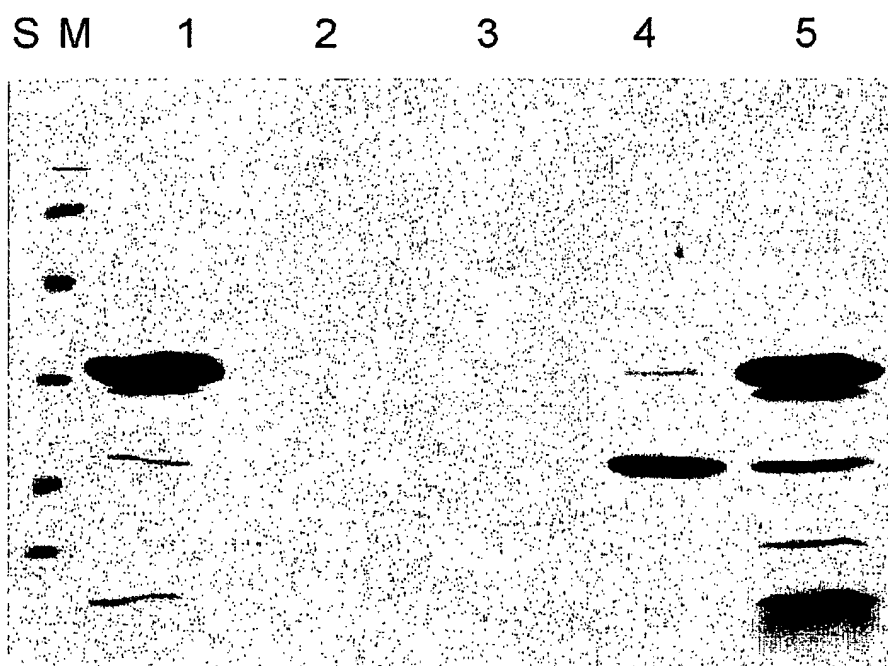
S M 1 2 3 4 5 6



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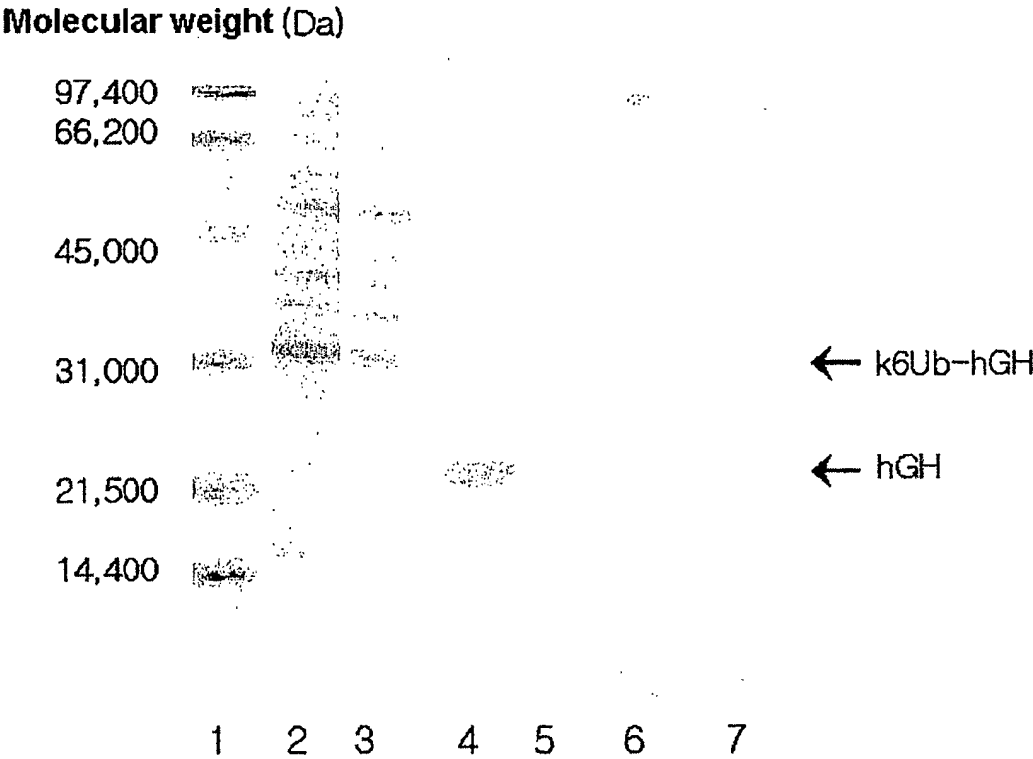
**FIG.5**

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**FIG.6**

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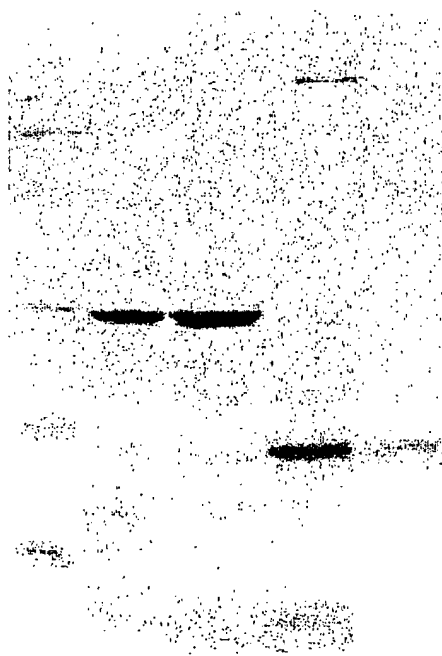
FIG.7



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**FIG.8**

SM 1 2 3 4

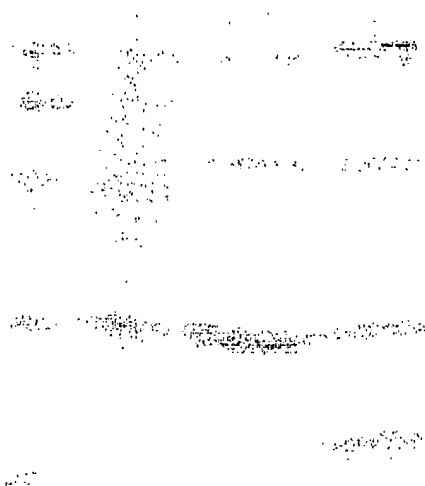




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**FIG.9**

S M	1	2	3	4
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# INTERNATIONAL SEARCH REPORT

International application No.

PCT/KR02/01416

## A. CLASSIFICATION OF SUBJECT MATTER

**IPC7 C07K 19/00**

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC7 C07K 19/00

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Korean Patents and applications for inventions since 1975

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CAPLUS, BIOSIS, MEDLINE, WPINDEX

"ubiquitin and (fusion or conjugat\* or hybrid or chimeric)"

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 9423040 A1 (CELTRIX PHARMACEUTICALS, INC) 13 OCTOBER 1994	1 - 4
A	US 5914254 A (CELTRIX PHARMACEUTICALS, INC) 22 JUNE 1999	1 - 4
A	GB 0026484 A0 (ABEZON SILVA et al.) 13 DECEMBER 2000	3
A	US 6068994 A (CHIRON CORPORATION) 30 MAY 2000	14
A	US 5196321 A (MASSACHUSETTS INSTITUTE OF TECHNOLOGY) 23 MARCH 1993	5 - 13

☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

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Date of the actual completion of the international search

25 NOVEMBER 2002 (25.11.2002)

Date of mailing of the international search report

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## INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/KR02/01416

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